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P26 – calcium binding protein from bovine retinal photoreceptor cells

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The primary structure of bovine retinal calcium binding protein P26 has been determined by parallel analysis of protein and corresponding cDNA. This protein is identical to recovering and shares 59% homology with visinin, a cone specific calcium binding protein from chicken retina. Preliminary data are presented on expression of P26 as a fusion protein in *E. coli*.

P26; Calcium binding; Primary structure; Nucleotide sequence; Expression

1. INTRODUCTION

Decrease of intracellular calcium concentration is one of the consequences of photoactivation of vertebrate photoreceptor rod outer segments (ROS). This occurs as a result of closure of a cGMP gated cation channel in the plasma membrane and continuous operation of Na^+,K^+,Ca^{2+}-exchanger [1–3]. Data accumulated show that this decrease of calcium concentration triggers a negative feedback mechanism of the dark state restoration and light adaptation [4–6]. Electrophysiological and biochemical experiments clearly demonstrate that calcium concentration exerts its effect on photoreceptor guanylate cyclase such that lowered intracellular calcium levels result in increased synthesis of cGMP [7–11].

Rod outer segment cGMP-phosphodiesterase is another target sensitive to changes of intracellular calcium concentrations [12,13]. Recently it has been shown that calcium, in the physiological range, regulates the light activation of frog photoreceptor phosphodiesterase (PDE) [14]. The high sensitivity of both guanylate cyclase and phosphodiesterase to calcium is conferred by 26 kDa soluble proteins found in bovine and frog retina, respectively.

Detailed information both on protein and DNA levels is necessary for further structure–functional studies of this protein. As a first step in this direction we have isolated and cloned a cDNA encoding a 26 kDa protein of bovine retinal rod, designated P26. Preliminary data are presented on the expression of P26 in *E. coli* as a fusion protein.

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2. MATERIALS AND METHODS

Rod outer segments (ROS) were isolated from bovine retina according to procedure developed by Uhl [15]. The fraction of soluble proteins was obtained by resuspending ROS from 300 retinas in 200 ml of 20 mM HEPES, pH 7.2, containing 1 mM EDTA and 100 mM NaCl, followed by centrifugation at 29 000 × *g* (Ti-19, Beckman Instruments) for 1.5 h. Preparation of ROS and extraction of soluble proteins were carried out in the dim red light at 4°C. Supernatant was concentrated by Amicon YM-10 filtration, dialysed against 1.5 liters of sample buffer (50 mM Tris-HCl, pH 7.4, containing 1 mM $CaCl_2$ and 100 mM NaCl) and loaded on a Fluorosorb column (0.5 × 10 cm). (Fluorosorb is available from the Shemyakin Institute of Bioorganic Chemistry.) Prior to sample application the column was washed with 15 ml of 0.1% BSA and then equilibrated with the sample buffer. The ROS extract was applied onto the column and the column was developed with the same buffer at a flow rate 20 ml/h until the A_{280} returned to baseline. Then it was equilibrated in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA. The bound proteins were eluted with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% octylglucoside (OG). The final purification of P26 was carried out on a Mono-Q column (HR 5/5; Pharmacia Fine Chemicals) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA. Linear gradient of KCl (0–200 mM) at a flow rate 0.5 ml/min was used to elute proteins. Aliquots (20 μl) were analysed by SDS-PAGE.

Purified P26 was cleaved with 100-fold excess of CNBr per mole of methionine in 50% CF_3COOH for 24 h at room temperature. The peptides were first fractionated on a Bio-Gel P-10 (1 × 100 cm) column equilibrated with 20% CH_3COOH and further purified by RP-HPLC (Zorbax C8, C18) on Altex model 332 system (Altex, USA). The column was developed with a linear gradient of acetonitrile (0–80%) in 0.1% CF_3COOH.

Digestion of P26 with clostripain (arginine specific protease) at an enzyme-to-substrate ratio of 1:50 was carried out in 100 mM NH_4HCO_3, pH 7.7, 1 mM DTT, 16 h at room temperature. The resulting peptides were purified by RP-HPLC as indicated above.

Bovine retinal cDNA library in bacteriophage λZAP was the gift of Dr. M. Applebury (Department of Ophthalmology, University Chicago). DNA manipulations were carried out by using standard methods [16]. Nucleotide sequences were determined by Maxam and Gilbert method in a solid phase modification. Construction of plasmid encoding a fusion protein and expression of this protein in *E. coli* will be given in details in the accompanying paper.

3. RESULTS AND DISCUSSION

At least four different methods of P26 purification are now available. P26 was originally purified using an affinity chromatography column based on detergent solubilised ROS membranes coupled to concanavalin A-Sepharose [17]. Some methods are based on selective calcium-dependent binding of this protein to hydrophobic carriers (phenyl-Sepharose) or to ROS disk membranes [14,18]. Others take advantage of temperature-dependent aggregation and precipitation of the majority of ROS soluble proteins leaving P26 in the supernatant [19]. What is common for all these procedures is the use of ion-exchange chromatography as a final step of purification.

The procedure we have developed is based on the observation that Fluorosorb (a teflon-coated silica gel) selectively adsorbs P26 together with three proteins with molecular masses higher than that of P26. Our attempts to elute P26 from the column with EDTA or high ionic strength were unsuccessful. However, it can easily be eluted with lauryldimethylaminooxide (LDAO) or OG and finally purified by ion-exchange chromatography or gel-filtration. Purified protein migrates on SDS-PAGE as a single band with the molecular mass 26 kDa and gives a positive signal on calcium blots (Fig. 1).

Attempts to sequence intact protein were unsuccessful because of a blocked amino-terminus; the nature of this modification remains unknown. Cleavage at methionine with CNBr and digestion with clostripain were used for P26 fragmentation. CNBr peptides were first fractionated on Bio-Gel P-10 in acetic acid and finally purified using RP-HPLC in acetonitrile gradient. Peptides of the clostripain digest were directly applied to a Zorbax C8 or C18 column and eluted with acetonitrile gradient in 0.1% trifluoroacetic acid. The complete or partial amino acid sequences of 8 peptides comprising 60% of the polypeptide chain were determined [20]; all of them are underlined in Fig. 2.

Oligodeoxyribonucleotides of the following sequences

5'-AACACCAAGTTCACCGAGGAGGAGCT-3' (1)

5'-CACCTCCCCGAGGACGAGAACACCCC-3' (2)

corresponding to peptides

Asn-Thr-Lys-Phe-Thr-Glu-Glu-Glu-Leu (1)

His-Leu-Pro-Glu-Asp-Glu-Asn-Thr-Pro (2)

respectively, were synthesised taking into account the codon usage of several known ROS proteins and used as hybridization probes to screen retinal cDNA library in bacteriophage λZAP. Five hybridization-positive clones were isolated from 1×10^6 transformants. Three of them: pSKP26-2 (−), pSKP26-4 (−) and pSKP26-5 (−) were used for sequence analysis of cloned inserts (Fig. 2).

The results of the sequence analysis demonstrated that none of these clones contains a full-size cDNA of P26. Several base pairs of the 5'-coding sequence were missing in the first two inserts, but they contained the rest of the coding sequence with 3' untranslated region including polyadenylation signal (AATAAA) as well as polyA sequence, 17 base pairs downstream of this signal. In the third clone, on the contrary, 90 base pairs were missing from the 3'-coding sequence but it contained 61 base pairs upstream of the initiation codon.

Fig. 3 shows the 606 nucleotide sequence of P26 cDNA. All the complete and partial amino acid sequences determined by peptide analysis were found to be encoded by the cDNA sequence in the same reading frame. Triplet ATG (62–64) is the initiation codon since it is the first ATG codon that appears downstream of a nonsense codon (32–34) found in the frame. The codon 202 specifying leucine is followed by the termination codon. Thus, the P26 amino acid sequence deduced from the corresponding cDNA nucleotide sequence consists of 202 residues with the molecular mass 23.3 kDa.

The amino acid sequence of bovine retinal calcium binding protein, designated recoverin, has been recently determined mainly at the protein level [21]. The results of this study are in complete agreement with our data on P26. However, no information was given as to the nucleotide sequence of the corresponding full-length cDNA of recoverin, which is important for further

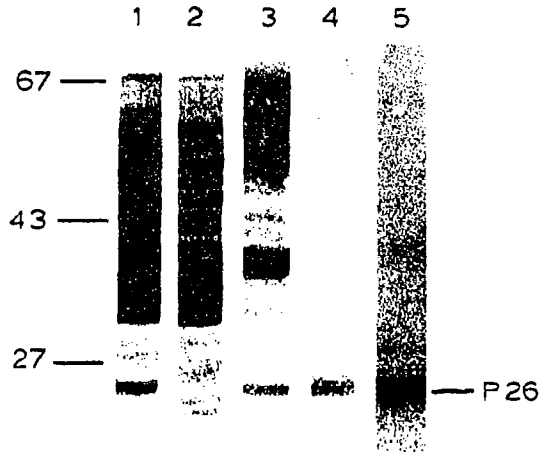

Fig. 1. Purification of bovine retinal P26. Soluble fraction of ROS proteins in 50 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 100 mM NaCl was applied to a Fluorosorb column. An aliquot of soluble fraction was separated on SDS-PAGE (lane 1). Proteins which did not bind to the column (lane 2). Bound proteins which could be eluted with octylglucoside (lane 3). The eluted proteins shown in lane 3 were further purified using ion exchange chromatography on Mono-Q (lane 4). Calcium blot of the purified P26 (lane 5). Molecular weight standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (27 kDa).

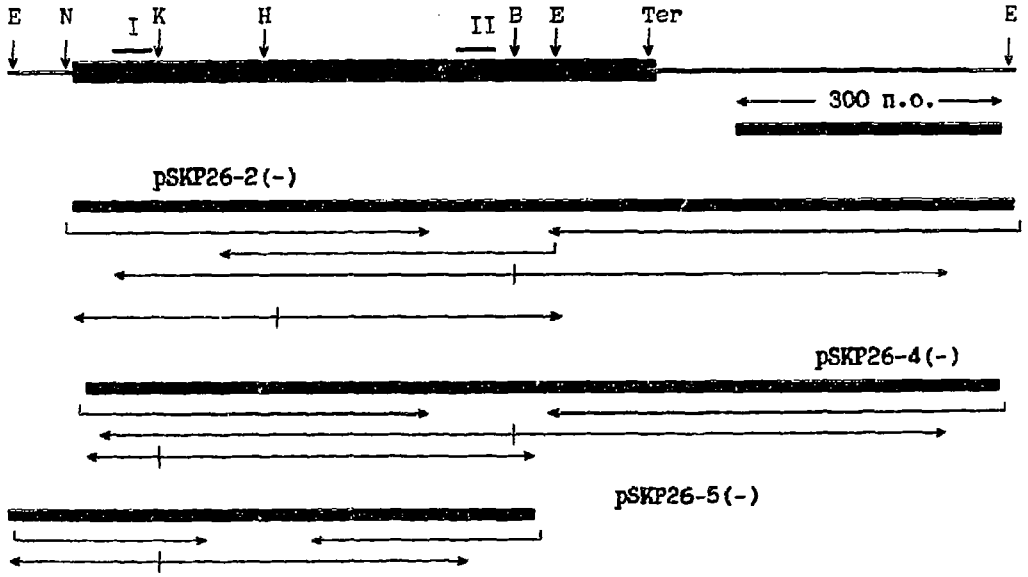

Fig. 2. Location of inserts of isolated clones in the restriction map of P26 cDNA and sequence strategy. The solid boxes indicate the coding regions. The arrows under the cDNA fragments indicate the extent and direction of sequence determination. B = *Bgl*II; E = *Eco*RI; H = *Hin*dIII; K = *Kpn*I; N = *Nco*I. I and II indicate nucleotide probes used for screening of cDNA library.

structure–functional studies using recombinant DNA technique.

At the early stage of this investigation we found that several peptide sequences of P26 show a high homology to troponin C, chicken vitamin D-dependent calcium binding protein and calmodulin. When the sequences analysed later were compared with the NBRF Protein Identification Resource, the highest homology scores observed were with visinin, a chicken retinal cone and pineal gland specific calcium binding protein [22]. Taking into account light-dependent increase of visinin expression in pineal gland and cultured pineal bodies it was suggested that this protein is involved in the phototransduction mechanism of cone and pineal cells. The amino acid sequences of visinin (192 residues) and P26 share 59% homology; the lowest variability is observed in the central parts (extending from amino acid residue 65–125) of respective polypeptide chains.

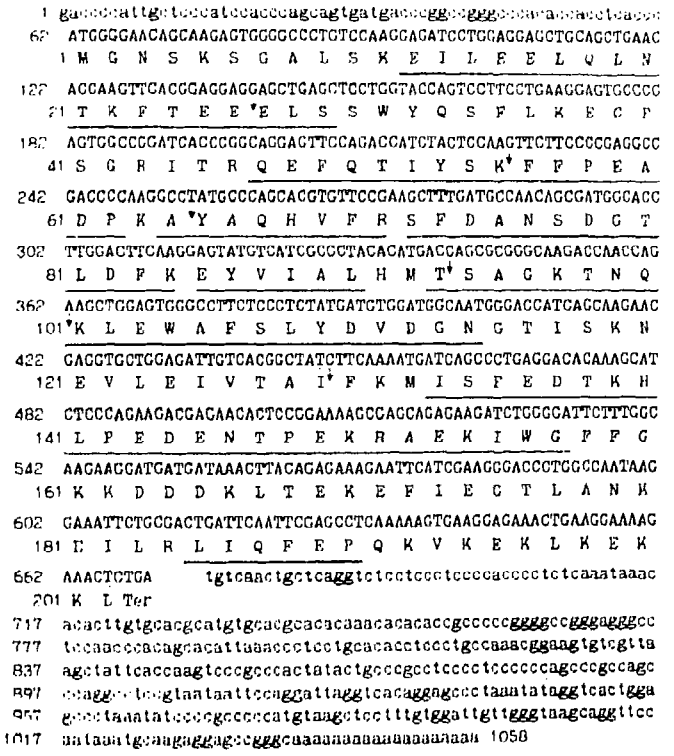

Fig. 3. Nucleotide and deduced amino acid sequence of bovine retinal protein P26. Sequences determined by peptide analyses as well as polyadenylation signal are underlined. Arrows indicate EF-hand motifs. Ter. = translation termination signal.

Both visinin and P26 contain three consensus calcium binding sites of the EF-hand motif (Fig. 3, dashed lines). In accordance with the known criteria [23], each EF-hand comprises 29 amino acid residues and is made of two α-helices with the intermediate sequences containing calcium coordinating amino acid side chains. The only deviation is the insertion of cysteine residue at position 39 in the first EF-hand. Whether this cysteine residue is inserted to diminish the calcium binding potential of the first EF-hand so as to provide a fine regulation of some unknown function remains to be seen. In any case, this cysteine might be a good candidate for site directed mutagenesis.

Recoverin or P26 was shown to activate ROS guanylate cyclase when the free calcium concentration is lowered from 450 to 40 nM [21]. This accords with the previous observations that the decrease in calcium concentration enhances guanylate cyclase activity during the restoration phase [17].

Retinal calcium binding protein may also exercise other functions. In this context two recent observations

deserve attention. First, a calcium binding protein of the molecular mass 26 kDa homologous to chicken visinin was purified from frog ROS. It was shown that in the presence of 1 μM calcium this protein, designated *S*-modulin [14], increases the efficiency of PDE activation by 50% without affecting the maximum activity. Without *S*-modulin, PDE activation was not markedly influenced by either low (30 nM) or high (1 μM) calcium concentrations. It would be interesting to know if *S*-modulin and P26 are switched to phosphodiesterase and guanylate cyclase, respectively, or each of these proteins has a regulatory effect on both enzymes. Second, the sera from patients with cancer-associated retinopathy (CAR) label a 26 kDa protein from human retina [18]. Comparison of amino acid sequences demonstrates that CAR antigen is the same protein as P26.

Taken together, it is difficult to overestimate the importance of the role P26 plays both in our understanding of basic problems of vision and disorders in retina. Now that the cDNA of P26 is available, wide perspectives are open for detailed structure–functional studies of this protein using recombinant DNA technique. As a first step in this direction we have expressed P26 in *E. coli* as a fusion protein (*St. aureus* protein A fragment–P26) [24]. The advantage of this expression system is that it provides the secretion of a fusion protein, facile purification using the affinity chromatography on IgG-Sepharose and cleavage at enteropeptidase specific site located between the protein A fragment and P26. Finally, the yield of a fusion protein is high enough to carry out extensive structural and functional studies of P26.

REFERENCES

[1] Yan, K.W. and Nakatani, K. (1984) Nature 3116, 352–354.
[2] Cervetto, L., Lagnado, L., Perry, R.J., Robinson, D.W. and McNaughton, P.A. (1989) Nature 337, 740–743.
[3] Yan, K.W. and Nakatani, K. (1985) Nature 313, 579–582.
[4] Matthews, H.R., Murphy, R.M., Fain, G.L. and Lamb, T.D. (1988) Nature 337, 67–69.
[5] Nakatani, K. and Yan, K.W. (1988) Nature 334, 69–71.
[6] Pugh Jr., E.N. and Lamb, T.D. (1990) Vision Res. 30, 1923–1948.
[7] Hodgkin, A.L. and Nunn, B.J. (1988) J. Physiol. 403, 439–471.
[8] Rispeli, G., Sather, W.A. and Detwiler, P.B. (1988) Biophys. J. 53, 388a.
[9] Kawamura, S. and Murakami, M. (1989) J. Gen. Physiol. 94, 649–668.
[10] Pepe, J.M., Panfoli, J. and Cugnoli, C. (1986) FEBS Lett. 203, 73–76.
[11] Koch, K.W. and Stryer, L. (1988) Nature 334, 64–66.
[12] Robinson, P.R., Kawamura, S., Abramson, B. and Bownds, M.D. (1980) J. Gen. Physiol. 766, 631–645.
[13] Barkdoll, A.E., Pugh, E.N. and Sitaramayya, A. (1989) J. Gen. Physiol. 93, 1091–1108.
[14] Kawamura, S. and Nakamura, M. (1990) Nature 349, 420–423.
[15] Uhl, R. (1987) J. Biochem. Biophys. Methods 14, 127–138.
[16] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual Cold Spring Harbor Lab., Cold Spring Harbor, New York, NY.
[17] Dizhoor, A.M., Nekrasova, E.R. and Philipov, P.P. (1989) Biochem. Biophys. Res. Commun. 162, 544–547.
[18] Polans, A.S., Buczylko, J., Crabb, J. and Polczewski, K. (1991) J. Cell. Biol. 112, 981–989.
[19] Lambrecht, H.-G. and Koch, K.-W. (1991) EMBO J. 10, 793–798.
[20] Zung, N.T., Klezovitch, O.N., Kutuzov, M.A. and Levina, N.B. and Abdulaev, N.G. (1991) Sensory Systems 5, 14–19 (Russ.).
[21] Dizhoor, A.M., Ray, S., Kumar, S., Niem, C., Spengler, M., Brolley, D., Walsh, K., Philipov, P.P., Hurley, J. and Stryer, L. (1991) Science 251, 915–918.
[22] Yamagata, K., Goto, K., Kuo, Ch-H., Kondo, H. and Niki, N. (1990) Neuron 2, 469–476.
[23] Moncrief, N.D., Kretsinger, R.H. and Goodman, M. (1990) J. Mol. Evol. 30, 522–562.
[24] Shmukler, B.E., Kutuzov, M.A., Zargarov, A.A. and Abdulaev, N.G. (1991) FEBS Lett. (in press).